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The Need for Selenite and Molybdate in the Formation of Formic Dehydrogenase by Members of the *Coli-aerogenes* Group of Bacteria

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Factors required in trace amounts for growth are generally considered to play a catalytic role in the metabolism of the organism, and comparison of the enzymic activity of normal and deficient cells has yielded information concerning the function of organic growth factors in the metabolism of micro-organisms. Waring & Werkman (1944) have used this method to study the function of iron in *Aerobacter indologenes*; they found that the activity of formic dehydrogenase, among other enzymes, was greatly reduced in iron-deficient cells. It does not follow, however, that only such inorganic factors as are required for normal growth are concerned in the formation of enzymes, since an enzyme whose function is not indispensable to the anabolism of the cell could be greatly reduced in amount without any effect on growth being apparent. This paper describes an instance of this sort; traces of selenite and molybdate, as well as iron, were found to be essential for the formation of an active formic dehydrogenase by strains of coliform organisms grown in a purified synthetic medium, although the first two factors, i.e. selenite and molybdate, had no influence on growth.

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EXPERIMENTAL

Organisms. That most commonly used was 1433, a strain of *Escherichia coli* obtained from Dr M. R. Pollock. Essentially similar results were obtained using two other strains, N.C.T.C. no. 86, and *Esch. coli* S from the collection of Dr C. B. van Niel, and two strains of *Aerobacter aerogenes*, N.C.T.C. no. 5936, and *Aero. aerogenes* 1, supplied by Dr E. F. Gale.

Purification of chemicals. Since conflicting results were obtained in the early experiments, in which the 8-hydroxyquinoline extraction technique described by Waring & Werkman (1944) was used, a more rigorous method of purification was resorted to. The individual chemicals employed in the preparation of the media were treated as described below. The techniques were designed so as to eliminate traces of impurities by distillation where possible, and also to make use of the capacity of $Fe(OH)_3$ to adsorb traces of selenite among other ions.

Certain general precautions were taken in all manipulations. Only Pyrex glassware was used; each item carefully cleaned immediately before use by rinsing first with ethanolic KOH, followed by distilled water, conc. HCl, and finally with glass-distilled water. Care was taken throughout to keep beakers and the necks of flasks covered with hardened filter paper or with cleaned beakers to avoid contamination with dust. The water and solvents used during the purifications were freshly distilled in an all-Pyrex still.

The purified chemicals were stored in cleaned, wide-mouthed Pyrex bottles, and kept in a clean desiccator to

avoid contamination. Stock solutions of $MgSO_4$, $FeSO_4$, H_2SeO_3 and Na_2MoO_4 were kept in Pyrex bottles protected from dust, and were made up freshly every 3 or 4 weeks as an additional precaution.

Ammonium dihydrogen phosphate. This was prepared from redistilled ammonia and phosphorus oxychloride as suggested by Hutner, Provasoli, Schatz & Haskins (1950). The salt was recrystallized from water, filtered off and dried at 100°.

Potassium chloride. 20 g. KCl were dissolved with heating in 55 ml. water containing 0·5 ml. 50% (w/v) $FeCl_3$. Approximately 6 N redistilled NH_4OH was added until a precipitate of $Fe(OH)_3$ had been formed and the solution was alkaline. The precipitate was removed, and the solution shaken, while still warm, with 8-hydroxyquinoline in $CHCl_3$ solution to remove any trace of unprecipitated iron. After further extractions with portions of clean $CHCl_3$ to remove residual 8-hydroxyquinoline, the solution was cooled to a low temperature; the crystals were collected by filtration and dried at 100°.

Other chemicals. $NaNO_3$ and sodium succinate were purified by similar means; the latter was dried *in vacuo*. Glycerol was redistilled under reduced pressure. $MgSO_4$, $FeSO_4$ and ammonium molybdate were recrystallized. SeO_2 was prepared from selenium metal as described by Baker & Maxson (1939), and purified by sublimation. It dissolved readily in water to form selenious acid, which was not neutralized.

No special purifications of the substances investigated for ability to replace selenite and molybdate were undertaken. Analytical grade samples of NH_4VO_3, $K_2Cr_2O_7$, Na_2WO_4 and $Na_2SeO_4.10H_2O$ were used, while K_2TeO_3, $(NH_4)_2TeO_4$ and $UO_2(NO_3)_2.6H_2O$ were standard laboratory reagents.

Growth medium. The precise composition of the growth medium had no influence on the need for the presence of selenite and molybdate for the production of an active formic dehydrogenase. Thus neither wide variation of the concentration of inorganic constituents nor the use of different sources of carbon and energy had any effect on the results. Glucose, glucose/DL-lactate, succinate/L-glutamate and succinate/glycerol were investigated as carbon and energy sources. The medium generally used contained only easily purified substances and the concentrations of these were the least required to yield about 1 mg. dry cells/ml. under the conditions employed. Its composition was as follows: $(NH_4)H_2PO_4$, 0·0773 g.; KCl, 0·015 g.; sodium succinate, 0·2 g.; glycerol, 0·1 ml.; $MgSO_4.7H_2O$, 5·07 mg. ($\equiv$500 μg. Mg^{2+}); $FeSO_4.7H_2O$, 5 μg. ($\equiv$1 μg. Fe^{2+}); made up to 100 ml. with fresh, glass-distilled water. When added, both molybdate and selenite were at 1×10^{-7} M concentration.

In spite of failing to provide many trace elements, this medium appeared to be complete. Neither the growth nor the activity of cells was affected by the further addition of copper, cobalt, manganese and zinc. On the other hand, omission of iron resulted in a 100-fold reduction in cell yield, and omission of magnesium in at least a 1000-fold reduction (no visible growth was obtained).

Experiments were also carried out in which a nitrate-containing medium was used; it contained the above-mentioned minerals, but a higher $(NH_4)H_2PO_4$ content (0·4%, w/v) to raise its buffering capacity, 0·5% (w/v) $NaNO_3$, and 0·05% (w/v) glucose as carbon and energy source.

Growth conditions. The nitrate-free medium was made up immediately before use, and dispensed in 50 ml. lots in 500 ml. Erlenmeyer flasks. Appropriate quantities of selenite and molybdate solutions were added to each flask and the necks of the flasks were covered with suitably sized beakers. A layer of cotton wool was tied so as to cover the gap between the rim of the beaker and the neck of the flask. The inoculum was prepared by suspending a loopful of the growth from an 8 hr. 5% (v/v) yeast-autolysate agar slope in sterile, glass-distilled water, and one drop of this was added to each flask after autoclaving. The flasks were incubated on a horizontal shaker at 28–30°.

The nitrate-containing medium was made up in a similar way, but dispensed in 100 ml. lots in 200 ml. Erlenmeyer flasks. The cultures were also incubated at 28–30°, but were not shaken.

Preparation of washed suspensions. The cells were harvested at the point at which the cultures attained their maximum density, which was usually 15–20 hr. after inoculation. This was not strictly necessary for cultures grown in the nitrate-free medium, in which, as shown by Wooldridge, Glass & Knox (1936), the formic dehydrogenase activity remains roughly constant for a considerable period after growth has ceased. With the nitrate-containing medium, however, a peak in activity coincident with the cessation of growth was observed; thereafter the decay of formic dehydrogenase activity was rapid.

The cells were washed once in distilled water. This was found to be sufficient to avoid high blank O_2 uptakes and it was not found necessary to use glass-distilled water, or to take special precautions to prevent contamination with dust during washing, in order to demonstrate the effect of selenite or molybdate deficiency in the growth medium on the formic dehydrogenase activity of the cells. Waring & Werkman (1944) made a similar observation in their experiments on iron deficiency. The suspensions were adjusted to a concentration of 5 mg. dry wt./ml., using a Hilger or a Klett-Summerson Photoelectric Absorptiometer previously calibrated against suspensions of known dry wt.

Estimation of formic dehydrogenase activity. The rate of O_2 consumption in the presence of formate was taken as an index of the formic dehydrogenase activity of the cells (cf. Gale, 1939); no special precautions as to the purity of the reagents or cleanliness of the glassware were taken. Warburg manometers were used, the cups containing: cell suspension, 1 ml.; 0·1 M-KH_2PO_4/Na_2HPO_4 buffer, pH 6, 0·5 ml.; 0·5 M sodium formate (side bulb) 0·1 ml.; 20% (w/v) KOH (centre well) 0·2 ml.; water, 0·2 ml. The gas phase was air, and the temp. 30°. The rate of O_2 consumption in the absence of added substrate was low, and remained roughly linear for a period of 1 hr. In practice, therefore, the blank O_2 uptake was measured for 10 or 15 min. after equilibration, and the formate then tipped into the main compartment of the cup. O_2 consumption in the presence of substrate reached its highest rate after about 5 min., and was followed for 30–40 min.; it remained virtually linear for a period of at least 1 hr. at this temp. in contrast to the falling rate noted by Gale (1939) at 37°. O_2 uptake in the presence of glucose was measured similarly, except that the buffer used had a pH of 7.

On occasion, formic dehydrogenase was also measured by determining the rate of methylene-blue reduction in evacuated Thunberg tubes, whose contents were as follows: cell suspension, 0·5 ml.; 0·1 M-KH_2PO_4/Na_2HPO_4 buffer,

pH 6, 1 ml.; water, 0·3 ml.; 0·5 M sodium formate (stopper), 0·1 ml.; 0·002 M methylene blue (stopper) 0·1 ml.

The results for blank O_2 uptakes, and for those in the presence of substrate (uncorrected for blank) are expressed as Q_{O_2} (μl. O_2 taken up/hr. by 1 mg. dry wt. of cells), or as Q_{MB} (μl. methylene blue reduced/hr. by 1 mg. dry wt. of cells, where 1 μmole = 22·4 μl.). Thus Q_{O_2} is equivalent to 0·5 Q_{MB}.

Cell-free preparations. These were made by tryptic digestion of the cells (Gale, 1939), and grinding with powdered glass.

RESULTS

Identification of factors needed to obtain active cell suspensions. This investigation arose from the observation that confirmation of the need for iron in formic dehydrogenase formation, demonstrated by Waring & Werkman (1944), could only be obtained if a mineral supplement containing copper, cobalt, manganese, zinc and molybdenum were added to the medium. In these experiments the nitrate-containing medium was used, purified by extraction with 8-hydroxyquinoline. Cells harvested from such media without the addition of the above-mentioned mineral supplement had a Q_{O_2} in the presence of formate of −20 or less, regardless of the amount of iron in the medium. If the supplement had previously been added, however, cells grown in the presence of sufficient iron had a Q_{O_2} with formate of about −100, and the effect of iron deficiency was dramatic. Hence it seemed clear that the supplement contained an ingredient which was required for the synthesis of formic dehydrogenase in the presence of adequate iron. This was identified as molybdate.

When the batches of chemicals used in these tests had been exhausted, even these results could no longer be duplicated except when the medium was made up with tap water. It was inferred, therefore, that some second factor, present in London tap water, and previously added to the medium as an impurity in the chemicals used, was involved in formic dehydrogenase formation. Attempts to isolate this from tap water were not successful. However, by treating boiler-scale with nitric acid, a solution in water was obtained that effectively replaced tap water as a means of inducing formic dehydrogenase synthesis, and by standard analytical procedures it was possible to show that the specific component belonged to the group of elements precipitated by hydrogen sulphide in acid solution, namely, bismuth, copper, cadmium, gold, platinum, mercury, arsenic, antimony, tin, molybdenum, selenium and tellurium. Solutions containing these elements were prepared, and selenite was found to be the only substance having any effect on formic dehydrogenase synthesis. It was clear by this stage that the procedure of extracting with 8-hydroxyquinoline was inadequate for purification of the medium, and the preparation of carefully purified reagents was undertaken. Results given below refer only to experiments in which media made up with such chemicals were used.

Experiments in purified media. Cells grown in a medium made with purified ingredients possessed a high formic dehydrogenase activity only if both selenite and molybdate were added, and sufficient iron had been supplied (Table 1). In the experiments described below, a medium containing 0·01 μg. Fe^{2+}/ml. was therefore used. In the great majority of tests neither selenite nor molybdate alone caused any increase in the formic dehydrogenase activity of the cells; therefore in the few instances where such an increase in activity was observed in the presence of only one of the two substances the effect was attributed to contamination of the medium or the glassware with a trace of the

Table 1. *Effect of inorganic factors in growth medium on formic dehydrogenase activity of* Esch. coli 1433

The manometer cups contained: cell suspension, 1 ml.; 0·1 M sodium plus potassium phosphate buffer, pH 6 for formate, pH 7 for glucose, 0·5 ml.; 0·5 M sodium formate or glucose (side bulb) 0·1 ml.; 20% (w/v) KOH (centre well) 0·2 ml.; water 0·2 ml.; gas phase, air; temp. 30°.

The Thunberg tubes contained: cell suspension, 0·5 ml.; 0·1 M sodium plus potassium phosphate buffer, pH 6, 1 ml; water, 0·3 ml.; 0·5 M sodium formate (stopper) 0·1 ml.; 0·002 M methylene blue (stopper) 0·1 ml. For further details see text.

Growth medium	Factors added to growth medium	Final growth (mg./ml.)	Blank $-Q_{O_2}$	$-Q_{O_2}$ glucose	$-Q_{O_2}$ formate	$\frac{Q_{MB}}{2}$ formate
Nitrate-free	Mg^{2+}	0·011	5	—	31	—
	Mg^{2+}, SeO_3^{2-}, MoO_4^{2-}	0·012	6	—	20	—
	Mg^{2+}, Fe^{2+}	0·92	10	108	12	—
	Mg^{2+}, Fe^{2+}, SeO_3^{2-}	0·91	8	—	18	—
	Mg^{2+}, Fe^{2+}, MoO_4^{2-}	0·96	5	—	15	—
	Mg^{2+}, Fe^{2+}, SeO_3^{2-}, MoO_4^{2-}	0·93	3	114	116	—
Nitrate-containing (0·06 M)	Mg^{2+}	0·051	5	—	8	—
	Mg^{2+}, SeO_3^{2-}, MoO_4^{2-}	0·046	4	—	10	—
	Mg^{2+}, Fe^{2+}	0·14	5	96	8	—
	Mg^{2+}, Fe^{2+}, SeO_3^{2-}	0·16	3	114	30	25
	Mg^{2+}, Fe^{2+}, MoO_4^{2-}	0·15	7	112	16	19
	Mg^{2+}, Fe^{2+}, SeO_3^{2-}, MoO_4^{2-}	0·15	2	102	108	112

other factor. That this should occur occasionally, in spite of the precautions taken, is not surprising in view of the very low concentrations in which these factors are needed. Thus maximal stimulation in the nitrate free medium was obtained when the concentration of selenite was between 3×10^{-8} and 1×10^{-7} M, and that of molybdate about 1×10^{-8} M (Fig. 1). In the nitrate-containing medium, in which the yield of cells was 0·1–0·2 mg./ml., as compared with nearly 1 mg./ml., stimulation by molybdate in the presence of excess of selenite was detectable at a concentration as low as 1×10^{-10} M, and was maximal at 3×10^{-9} to 1×10^{-8} M.

The effect of selenite and molybdate was seen only if the cells were grown in the presence of these ions. Experiments were carried out in which cells grown in the presence of only one of the two factors were incubated in manometer cups for 45 min. at 30° with the other factor at a concentration of 1×10^{-3} M. This treatment was without effect; whereas the Q_{O_2} in the presence of formate was −18 and −20 for cells freshly harvested from media deficient in selenite or molybdate respectively, the corresponding values for the cells after incubation with the substance previously deficient were −17 and −15.

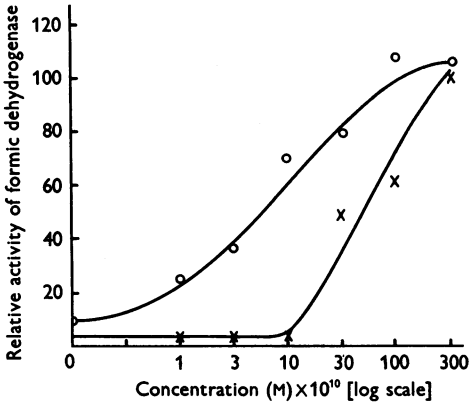

Fig. 1. Formic dehydrogenase activity of cells grown in media containing excess of either selenite or molybdate and varying amounts of the other substance. O—O, varying molybdate, selenite concentration $= 1 \times 10^{-7}$ M; ×—×, varying selenite, molybdate concentration $= 1 \times 10^{-7}$ M.

The rate at which cells took up oxygen if glucose were the substrate was approximately the same in all cases, and was independent of the formic dehydrogenase activity (Table 1).

Attempts to replace selenite and molybdate with other ions. As far as could be determined, the need for selenite and molybdate is highly specific, and no substitute was found.

Neither tellurate nor tellurite was able to replace selenite in the presence of excess of molybdate, even when their concentrations were 100 times that required for selenite to give full stimulation. Selenate had some activity at this concentration, but as the activity of several samples of selenate was raised to that of selenite by reduction on boiling in conc. hydrochloric acid, it is believed that the media to which selenate had been added contained some selenite as well, perhaps generated by a partial reduction of the selenate during autoclaving (Table 2).

Vanadate, chromate, tungstate and uranyl ions were tested for their capacity to replace molybdate in the presence of excess of selenite, but without success.

Effect of tungstate. Tungstate was found to have an action antagonistic to molybdate in the nitrate-containing medium, although no such effect was observed in the absence of nitrate. A strictly competitive relationship was observed between molybdate and tungstate if nitrate were present in the medium; the effect of molybdate was unaffected by 3 times, but annulled by 10 times its concentration of tungstate (Table 3). This relationship held good at least over the range 1×10^{-8} to 1×10^{-5} M. All the strains of *Esch. coli* that were used produce nitrite from nitrate during growth, and the antagonistic effect of tungstate was observed if the cells were grown in nitrate-free media to which 0·005M sodium nitrite had been added after autoclaving; in

Table 2. *Inability of other ions to replace selenite and molybdate*

Formic dehydrogenase activity was measured manometrically.

Excess of MoO_4^{2-} (10^{-7} M) present				Excess of SeO_3^{2-} (10^{-7} M) present			
Ion added	Concn. (M)	$-Q_{O_2}$	Relative activity	Ion added	Concn. (M)	$-Q_{O_2}$	Relative activity
SeO_3^{2-}	10^{-7}	107	100	MoO_4^{2-}	10^{-7}	113	100
—	—	15	14	—	—	16	14
TeO_3^{2-}	10^{-5}	8	7	VO_3^-	10^{-5}	15	13
TeO_4^{2-}	10^{-5}	6	6	$Cr_2O_7^{2-}$	10^{-5}	16	14
SeO_4^{2-}	10^{-7}	16	15	WO_4^{2-}	10^{-5}	14	12
SeO_4^{2-}	10^{-5}	60	56	UO_2^{2+}	10^{-5}	24	21
SeO_4^{2-} (after reduction) with HCl	10^{-7}	113	105				

Table 3. *Effect of tungstate and molybdate on formic dehydrogenase activity of* Esch. coli 1433

Formic dehydrogenase activity was measured manometrically and the results expressed as in Table 2.

Medium	WO_4^{2-} concn. (10^7 M)	MoO_4^{2-} concn. (10^7 M)	$-Q_{O_2}$	$\frac{WO_4^{2-}\text{ concn.}}{MoO_4^{2-}\text{ concn.}}$	Relative activity
Nitrate-free	0	1	135	—	100
	10	1	147	10	109
	100	1	127	100	94
Nitrate-containing (0·06 M)	0	0·1	98	—	100
	1	0·1	11	10	11
	1	0·3	77	3·3	78
	1	1	81	1	83
	10	0·1	6	100	6
	10	1	19	10	19
	10	3	94	3	96
	10	10	88	1	91
	100	0·1	11	1000	11
	100	100	125	1	128
Nitrite-containing (0·005 M)	0	1	157	—	100
	10	1	104	10	66
	100	1	28	100	16

this case a relatively higher concentration of tungstate was needed to neutralize the molybdate (Table 3). Tungstate (1×10^{-3} M) exerted no toxic effect on formic dehydrogenase if present in manometer cups during activity measurements.

Attempts to purify formic dehydrogenase. The isolation of the enzyme was attempted in order to elucidate the function of selenite and molybdate. It was found, however, in confirmation of Gale's (1939) results, that the enzyme was tenaciously bound to the cell fragments when the cells were broken by tryptic digestion or grinding with powdered glass. The only method found which appeared to bring the enzyme into solution was the butanol treatment described by Morton (1950); this, however, brought about such rapid inactivation that further purification of the enzyme was not achieved.

DISCUSSION

The effects of the deficiency of a far wider range of inorganic factors can be investigated if a medium prepared from specially purified chemicals is used, instead of relying on chelating reagents to remove unwanted ions from the solution. This method has not been widely used in the past, though Young, Begg & Pentz (1944), for example, used purified chemicals to investigate the inorganic growth requirements of *Esch. coli*. The technique used in the present investigation offered little difficulty, and once stocks of purified chemicals had been built up, the preparation of the medium took little more time than did that of the unpurified equivalent. Provided that proper care was taken to clean all glassware and to protect the medium at all stages from contamination with dust, consistent results were obtained if growth in the absence of iron were taken as index. Contamination with molybdate or selenite, reflected in relatively high Q_{O_2} values for cells grown with the addition of either factor alone, provides a more sensitive test of the reliability of the technique; in practice, such results were encountered only on three occasions.

The weight of a single cell of *Esch. coli* is approximately $2·5 \times 10^{-13}$ g. From this it can be calculated that the effect of molybdate on formic dehydrogenase activity in the presence of adequate selenite can be detected when only 50 molecules of it are available for each cell in the culture. Full activity is observed when each cell could contain 5000 molecules, though it is not, of course, certain that all the molybdate available in the medium finds its way into the cells. Rather larger numbers of selenite molecules are required, but full activity is developed when 5000 to 10000 molecules are present per cell. It seems most unlikely, therefore, that the effects observed could be due to impurities in the preparations of these two factors, and it is of interest that Burk (1934) calculated that the number of molybdenum atoms required per cell for maximum activity of the nitrogen-fixing mechanism of *Azotobacter vinelandii* was about 10000. While the need for molybdenum in nitrogen fixation has been recognized since the work of Bortels (1930), this is the first time that a biological function has been ascribed to selenium, although the presence of this element in the ash of plants has been known for many years. Moreover, the need for molybdenum in formic dehydrogenase formation differs from that in nitrogen fixation in that vanadium, shown by both Bortels (1930) and Burk (1934) to be capable of replacing molybdenum in the latter reaction, has no activity in stimulating the production of formic dehydrogenase.

The measurement of rates of oxygen uptake in the presence of some oxidizable substance is admittedly an indirect way of estimating the quantity of the responsible dehydrogenase in cells. In the present instance, however, it seems highly probable that the Q_{O_2} values obtained do reflect the enzyme content of the cells in view of the following considerations. First, the Q_{O_2} in the presence of glucose was always high, no matter what was the corresponding value for formate. This indicates that the mechanism for oxygen activation in the deficient cells was unimpaired. Secondly, Q values calculated from measurements of the rate of methylene-blue reduction in the presence of formate agreed well with those obtained from the manometric experiment. Finally, it was found that the activity of cell-free preparations corresponded to that of the parent cell suspension. As noted by Gale (1939), there was an apparent increase in formic dehydrogenase of up to double that of the intact cells on disruption of the cell membrane either by tryptic digestion or grinding with powdered glass when normal cells formed the starting material. With deficient cells, having a Q_{O_2} (formate) of about 10, very little or no increase of activity was observed on breaking the cells. These results argue in favour of the contention that the Q_{O_2} values represent an acceptable measure of the formic dehydrogenase content of the cells.

Though the work described above seems sufficient to establish that molybdate and selenite have an essential function in the formation of formic dehydrogenase, it is not at present clear whether these substances act as such, or undergo some combination before their use. Nor is it known whether they are eventually incorporated in the enzyme molecule, or are involved more remotely in its synthesis. Observation of the fact that selenite and molybdate influenced the formic dehydrogenase activity of cells only if these substances had been present in the growth medium, and were without effect in short-term experiments (in which cells deficient in only one of the substances were incubated with selenite or molybdate) does not provide a distinction between these possibilities, although it can be taken to indicate that neither of these substances forms a loosely bound part of an enzyme complex. This is in accord with Gale's (1939) demonstration that the activity of his cell-free preparation of formic dehydrogenase from *Esch. coli* was not affected by dialysis. The problem could be resolved if the enzyme could be purified, but in spite of intensive efforts no success in this direction has been achieved.

Billen & Lichstein (1951) have reported that the formic dehydrogenase activity of a strain of *Esch. coli* grown in a simple synthetic medium was low unless a mixture of amino acids were also present during growth. Stimulation of activity in washed suspensions incubated with the amino-acid mixture was also observed, although the unspecific nature of the procedure followed for estimation makes these experiments hard to interpret. It would appear to be possible, however, that their basic medium contained inadequate selenite or molybdate, and that the effect of the amino acids might be due, in part at least, to contamination with these inorganic factors. In experiments in which many samples of A.R. grade chemicals were used for preparing the medium, the results obtained could be explained in terms of various degrees of contamination with these two substances.

Billen (1951) has also reported an inhibitory effect of nitrate on formic dehydrogenase formation, and here the antagonism between molybdate and tungstate in nitrate-containing media might be operative. Why this antagonism should be apparent only if nitrate or some reduction product of nitrate is present in the medium is not immediately obvious. It might be suggested that in spite of their general similarity, free tungstate and free molybdate are too different to be interchangeable in a biological system; if nitrate is also present in the medium, however, a biologically active complex might be formed between molybdate and some derivative of the nitrate. This, being larger than the free ion, might be less readily distinguished from the corresponding tungstate complex, so that a typical case of competitive inhibition might become demonstrable.

SUMMARY

1. The preparation of a purified culture medium for *Escherichia coli* is described.

2. In such media, selenite and molybdate, as well as iron, are required for production of formic dehydrogenase.

3. These factors are effective only if present in the growth medium, and have no effect on washed suspensions.

4. Suspensions of cells oxidize glucose rapidly, whether they contain an active formic dehydrogenase or not.

5. Selenite is not replaceable by selenate, tellurite or tellurate.

6. Molybdate is not replaceable by vanadate, chromate, tungstate or uranyl ions.

7. If the medium contains nitrate, tungstate competitively antagonizes the effect of molybdate. Tungstate is without effect in nitrate-free media.

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Countercurrent Distribution Studies on Anterior Pituitary Growth Hormone

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Since the isolation of a growth hormone preparation which behaved as a homogeneous protein by electrophoretic, diffusion and solubility criteria (Li, Evans & Simpson, 1945), a number of biological activities have been attributed to this protein (for a review of these activities see Young, 1953). The question of whether or not the growth-promoting as well as the other activities are indeed properties of the protein itself, or whether they might reside in smaller molecules, becomes increasingly pertinent in view of the recent extensive work on the adrenocorticotrophic hormone and on the active principles of the posterior lobe of the pituitary gland.

Thus 'protein' adrenocorticotrophic hormone fulfils the same physical criteria of purity as growth hormone, but its biological activity can be separated from the bulk of the protein material by a variety of techniques (see Cortis-Jones, Crooke, Henly, Morris & Morris, 1950; Astwood, Raben, Payne & Grady, 1951; Dixon, Moore, Stack-Dunne & Young, 1951; Hess, Harris, Carpenter & Li, 1951). The same situation is found in the case of the posterior-pituitary principles. van Dyke, Chow, Greep & Rothen (1942) isolated a protein which contained pressor, antidiuretic and oxytocic activities in the same ratios as those of standard posterior-pituitary powder. This protein was homogeneous by ultracentrifugal and solubility criteria but showed some inhomogeneity by electrophoresis. Many workers, however, have been able to separate an oxytocic principle from an antidiuretic-pressor principle (see Kamm, Aldrich, Grote, Rowe & Bugbee, 1928; Irving & du Vigneaud, 1938; Potts & Gallagher, 1944), and both principles have recently been shown to be peptides of simple composition (Livermore & du Vigneaud, 1949; Pierce & du Vigneaud, 1950; Turner, Pierce & du Vigneaud, 1951).

In the present investigation, preparations of growth hormone have been subjected to countercurrent distribution in order to determine whether they would behave as a single substance under these conditions and to discover whether or not the growth-promoting activity could be separated from the bulk of the protein by this means.

EXPERIMENTAL

Growth-hormone preparations. Most of the growth hormone used in this investigation was prepared in this laboratory from ox pituitaries by a modification (Reid, 1952) of the method of Wilhelmi, Fishman & Russell (1948). The material thus prepared does not always appear homogeneous during electrophoresis at pH 9·5. In addition, 25 mg. of a preparation were kindly supplied by Prof. C. H. Li, Berkeley, California, U.S.A., to Prof. F. G. Young. It was stated that the material behaved as homogeneous when studied by zone electrophoresis, on a chromatographic column and by end-group analysis.

Counter-current distribution. The apparatus was a 54-tube, all-glass apparatus (Quickfit and Quartz Ltd.), similar to that described by Craig & Post (1949). The volume of each phase was 35 ml. The solvent system was 2-butanol : aqueous *p*-toluenesulphonic acid, the acid concentration being approximately 0·005 M or 0·0025 M. The system was prepared by equilibrating equal volumes of the acid and 2-butanol. The 2-butanol was redistilled and was low in peroxides when tested as described by Newton & Abraham (1950). Theoretical curves were constructed as described by Craig & Craig (1950). For distribution, the material was first suspended in 1–2 ml. of water and then treated with 33 ml. of the aqueous *p*-toluenesulphonic acid saturated with 2-butanol (lower phase). All preparations studied went completely into solution under these conditions. After the

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